Shape up your root

*Novel cellular pathways mediating root responses to salt stress and phosphate starvation*

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Chapter 3

Identification of downstream targets of SnRK2 action reveals a role for 5’ mRNA decay in root responses to salt stress

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Abstract

SNF1-RELATED PROTEIN KINASES 2 (SnRK2) are important components of early osmotic and salt stress signaling pathways in plants. The SnRK2 family comprises the abscisic acid (ABA) activated protein kinases SnRK2.2, SnRK2.3, SnRK2.6, SnRK2.7 and SnRK2.8, and the ABA-independent subclass 1 protein kinases SnRK2.1, SnRK2.4, SnRK2.5, SnRK2.9 and SnRK2.10. Subclass 1 SnRK2 are rapidly activated within the first minutes after application of salt stress in roots, but their mode of action, on the contrary to ABA-dependent SnRK2s, is not well understood. Here, we identified VCS (VARICOSE), a member of the mRNA decapping complex, as a direct substrate for phosphorylation by SnRK2.5, SnRK2.6 and SnRK2.10. Our results suggest that ABA-dependent and ABA-independent SnRK2 kinases act redundantly in mediating responses to salt stress by the regulation of 5’ mRNA decay. Transcriptome profiling of SnRK2 subclass 1 mutants revealed the aquaporin PIP2;5, and CYP79B2, coding for an enzyme involved in auxin biosynthesis, as putative genes acting downstream of SnRK2 subclass 1 protein kinases, possibly via 5’ mRNA decay. VCS and 5’-3’exoribonuclease XRN4 were shown to influence root responses to salinity. This study presents identification of the first substrate for SnRK2.4 and SnRK2.10. Phosphorylation of VCS and regulation of the transcription of genes involved in water transport, auxin biosynthesis or cellular signaling can shed some light on the ABA-independent SnRK2 protein kinases signaling pathway acting in responses to salinity.

Introduction

Soil salinity is one of the biggest constraints of modern agriculture, severely affecting crop productivity (Fita et al., 2015). Plant adaptation to saline conditions relies on early activation of signaling cascades which trigger protective mechanisms. Crucial components of salt and osmotic signaling pathways are protein kinases (Boudsocq and Lauriere, 2005). One group of protein kinases recognized as pivotal regulators of responses to osmotic stress is the plant specific SnRK2 family (SNF1-RELATED PROTEIN KINASE 2). Except for SnRK2.9, all other nine Arabidopsis SnRK2 protein kinase have been shown to have an increased kinase activity upon treatment with either ABA, osmotic stress or salt stress. However different responsiveness was observed for individual SnRK2 protein kinases (Boudsocq et al., 2004). The Arabidopsis SnRK2 protein kinase subfamily is comprised of three groups: subfamily 1 includes the abscisic acid (ABA) independent kinases (SnRK2.1, SnRK2.4, SnRK2.5, SnRK2.9, SnRK2.10), group 2 consists of these involved in drought responses (SnRK2.7 and SnRK2.8) and group 3 kinases are strongly activated by ABA (SnRK2.2, SnRK2.3, SnRK2.6; Kulik et al., 2011).
Members of the plant SnRK2 subfamily have also been identified in tobacco (Kelner et al., 2004), rice (Kobayashi et al., 2004), sorghum (Li et al., 2010), maize (Huai et al., 2008), wheat (Zhang et al., 2016), bean (Li and Assmann, 1996), soybean (Monks et al., 2001) and tomato (Yang et al., 2015).

Activity of SnRK2 protein kinases relies on their autophosphorylation, however activation by another protein kinase acting upstream has been also proposed (Boudsocq et al., 2007; Fujii et al., 2009). Two crucial residues, Ser171 and Ser175, have been found to be phosphorylated independently in the ABA-dependent SnRK2.6 protein, while activation of ABA-independent SnRK2.10 relied on sequential phosphorylation of Ser154 followed by phosphorylation of Ser158 (Vlad et al., 2010). Members of the ABA-dependent subclass 2 have been most extensively studied so far. SnRK2.2, SnRK2.3 and SnRK2.6 are components of the core ABA signaling pathway (Cutler et al., 2010). Several protein phosphatases from the PP2C clade A family have been shown to act as negative regulators of ABA-dependent SnRK2 kinases (Ma et al., 2009; Merlot et al., 2001; Nishimura et al., 2010; Park et al., 2009). In the absence of ABA, PP2Cs dephosphorylate SnRK2 to maintain their inactive state. ABA triggers the interaction of PP2Cs with ABA receptors PYR/PYL/RCAR (PYRABACTIN RESISTANCE 1/PYR1-LIKE/REGULATORY COMPONENT OF ABA RECEPTOR), thus releasing SnRK2 from their inhibited state (Ma et al., 2009; Merlot et al., 2001; Nishimura et al., 2010; Park et al., 2009). Members of subclass 2, SnRK2.7 and SnRK2.8, interacted with PP2Cs in vitro, while interactions in planta with one of the PP2Cs, ABI1 (ABSCISIC ACID INSENSITIVE 1), have been recently shown for SnRK2.8 and also the subclass 1 isoform SnRK2.4, but not for SnRK2.10 (Krzywinska et al., 2016; Umezawa et al., 2013; Vlad et al., 2010). SnRK2.4 and SnRK2.10 can be de-activated by members of the phosphoprotein phosphatase (PPP) family (Krzywinska et al., 2016). Activity of all ten SnRK2 protein kinases is negatively regulated by SCS (SnRK2-INTERACTING CALCIUM SENSOR; Bucholc et al., 2011). SnRK2.4 and SnRK2.10 have been shown to bind to the phospholipid second messenger phosphatidic acid, however the effect of this interaction on kinase activity remains unknown (Julkowska et al., 2015; Testerink et al., 2004).

Several downstream targets have been identified for SnRK2 subclass 2 and 3. SnRK2.6 phosphorylates the anion channel SLAC1, potassium channel KAT1, AtroH NADPH oxidases and aquaporin PIP2;1 thereby mediating ABA-dependent stomatal closure. A snrk2.6 knock-out mutant is impaired in closing stomata in low humidity conditions and has a wilting phenotype (Geiger et al., 2009; Grondin et al., 2015; Lee et al., 2009; Mustilli et al., 2002; Sato et al., 2009; Sirichandra et al., 2009; Yoshida et al., 2002). SnRK2.6 as well as SnRK2.2 and SnRK2.3 can phosphorylate ABA RESPONSIVE ELEMENTS-BINDING FACTORS AREB1, AREB2 and ABF3, bZIP transcription factors that bind to ABA-responsive elements in promoters of ABA-dependent genes (Fujii et al., 2007; Furihata et al., 2006). The
snrk2.2/snrk2.3/snrk2.6 triple mutant is insensitive to ABA and has low tolerance to drought, confirming the role of SnRK2 subclass 3 kinases as major regulators of ABA responses (Fujii and Zhu, 2009; Fujita et al., 2009). SnRK2 subclass 3 can also phosphorylate mitogen-activated protein kinases MPK1, MPK2 and MPK6, another class of ABA-activated protein kinases (Droillard et al., 2002; Umezawa et al., 2013; Wang et al., 2013). A recent phosphoproteomics study identified many novel putative SnRK2 subclass 3 targets that are involved in DNA and RNA binding and microRNA regulation, but their direct phosphorylation by these kinases still needs to be confirmed (Wang et al., 2013). SnRK2.8 phosphorylated ABF3 (redundant to SnRK2 subclass 2 proteins) and additionally targeted EEL, another ABF transcription factor (Mizoguchi et al., 2010). Moreover, SnRK2.8 phosphorylated three 14-3-3 proteins, adenosine kinase, glyoxylase 1 and ribose 5-phosphate isomerase, which links its function to the regulation of metabolic processes (Shin et al., 2007). Because the phosphorylation of SnRK2.4, SnRK2.10 or SnRK2.6 and two members of the mRNA decapping complex, VCS (VARICOSE) and DCP2 (DECAPPING2), was found previously in response to osmotic stress in two independent phosphoproteomic screens, it has been suggested that upon osmotic stress SnRK2 protein kinases could target the mRNA decapping complex (Kawa and Testerink, 2016; Stecker et al., 2014; Umezawa et al., 2013).

Targets of the SnRK2 subclass 1 subfamily remain still unknown. A screen using a semi-degenerate peptide array revealed dehydrins as putative SnRK2.10 substrates, but their direct phosphorylation was never confirmed (Vlad et al., 2008). SnRK2.4 and SnRK2.10 have been shown to be activated in Arabidopsis roots within 30 seconds of exposure to salt and may function as positive regulators of root growth under saline conditions (McLoughlin et al., 2012). snrk2.4 knock-out mutants showed a decreased main root length in the presence of salt, while snrk2.10 knock-out mutants exhibited reduced lateral root density (McLoughlin et al., 2012). Consistent with this phenotype, SnRK2.10 was expressed in the vasculature at the sites of lateral root formation, while SnRK2.4 was localized to most of the tissues in the main root (McLoughlin et al., 2012). Interestingly upon salt stress, SnRK2.4, but not SnRK2.10 re-localized to punctate structures of unknown identity (McLoughlin et al., 2012).

Here we describe the identification of novel interactors of the SnRK2.4 and 2.10 protein kinases, including proteins from the decapping complex and the mRNA degradation pathway. We show that SnRK2.10, as well as SnRK2.5 and SnRK2.6, can phosphorylate VCS, a member of the mRNA decapping complex. Using SnRK2 subclass 1 mutants, we have been able to identify several genes, whose salt-triggered change in expression is dependent on subclass 1 SnRK2 protein kinases. These include aquaporins PIP2;3 and PIP2;5, beta glucosidase BGLU6 and CYP79B2, coding for an enzyme involved in auxin biosynthesis. Further studies on the newly identified
interactors and the subclass 1 SnRK2-dependent salt responsive genes should help to understand the mode of action of ABA-independent SnRK2 protein kinases.

Results

SnRK2.4 and SnRK2.10 physically interact with proteins involved in mRNA metabolism

In order to identify putative up- and downstream SnRK2.4 and SnRK2.10 interactors, tandem affinity purification (TAP) using N- and C-terminal GS-rhino tag fusions of SnRK2.4 or SnRK2.10 expressed under control of a CaMV 35S promoter as baits was performed from Arabidopsis PSB-D cell suspension cultures (Van Leene et al., 2015). Commonly occurring proteins were treated as a background and were subtracted from the list of significant proteins (Van Leene et al., 2015). Eight proteins: VCS (VARICOSE), VCR (VARICOSE RELATED), DCP2 (DECAPPING 2), RRP44B (RRP44 HOMOLOG B), XRN4 (EXORIBONUCLEASE 4), SnRK2.7, PAT1H1 (TOPOISOMERASE II-ASSOCIATED PROTEIN and SnRK2.5 were co-purified with both SnRK2.4 and SnRK2.10, while AREB3 (ABA-RESPONSIVE ELEMENT BINDING PROTEIN 3) and SnRK2.6 were specific for SnRK2.4. ELP2 (ELONGATOR PROTEIN 2) and SnRK2.9 interacted only with SnRK2.10 (Table 1, Table S1). Interestingly, most of these putative interactors are proteins functioning in mRNA metabolism. VCS and DCP2 are part of the decapping complex, involved in removal of the 5’ mRNA cap and VCR, XRN4 and PAT1H1 have been previously shown to

Table 1. List of putative SnRK2.4 and SnRK2.10 interactors. Proteins identified by LC-MS/MS after TAP procedure from Arabidopsis suspension cultures. GSgreen N- and C-terminal fusions with SnRK2.4 and SnRK2.10 were used as baits with two technical replicates. Table presents proteins identified with at least 2 peptides for each sample.
colocalize in cytoplasmic protein foci called processing bodies (P bodies), which are the sites of mRNA degradation (Maldonado-Bonilla, 2014; Weber et al., 2008). The identified interactors are putative phosphorylation targets of SnRK2.4 and SnRK2.10. Peptides of identified SnRK2.4 candidates were searched for possible phosphorylation events in the purified complex. Eleven and six phosphopeptides were identified for VCS and VCR, respectively, but not for the other candidates, suggesting that VCS and VCR could be phosphorylation targets of SnRK2.4 and SnRK2.10 (Table 2).

**Table 2. Phosphorylation sites identified within the sequence of SnRK2.4 and its putative interactors identified via in planta TAP experiment.** Identified phosphorylated serines and tyrosines (pS, pT) are denoted in bold. For each peptide the localization in the protein is indicated with its start and end position. Sequences of peptides marked with shading were used for design of the synthetic peptides used for in vitro kinase activity assays.

<table>
<thead>
<tr>
<th>Protein name</th>
<th>Peptide start</th>
<th>Peptide end</th>
<th>Peptide sequence</th>
<th>Phosphorylation site position in protein sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>VCS</strong></td>
<td>86</td>
<td>100</td>
<td>TLSYPTPPLNLpSPPR</td>
<td>S98</td>
</tr>
<tr>
<td></td>
<td>142</td>
<td>156</td>
<td>SFPGGQPRPVPpACK</td>
<td>S154</td>
</tr>
<tr>
<td></td>
<td>637</td>
<td>662</td>
<td>TSGLPSQTSGAGAVLQpSPPR</td>
<td>S665</td>
</tr>
<tr>
<td></td>
<td>637</td>
<td>662</td>
<td>pTSGLPSQTSGAGAVLQpSPPR</td>
<td>T637+S665</td>
</tr>
<tr>
<td></td>
<td>637</td>
<td>666</td>
<td>TSGLPSQTSGAGAVLQpSPPR</td>
<td>Y651+S685</td>
</tr>
<tr>
<td></td>
<td>637</td>
<td>666</td>
<td>TSGLPSQTSGAGAVLQpSPPR</td>
<td>Y651+S685</td>
</tr>
<tr>
<td></td>
<td>690</td>
<td>699</td>
<td>TpgKADVSVDR</td>
<td>S692</td>
</tr>
<tr>
<td></td>
<td>821</td>
<td>835</td>
<td>VFCOSqVNLpSSTEMAR</td>
<td>S830</td>
</tr>
<tr>
<td></td>
<td>821</td>
<td>835</td>
<td>VFCQSVpNLSSTEMAR</td>
<td>S827</td>
</tr>
<tr>
<td></td>
<td>1149</td>
<td>1163</td>
<td>EMTSApSppSVAQALSR</td>
<td>S1155 or S1156</td>
</tr>
<tr>
<td></td>
<td>1171</td>
<td>1203</td>
<td>NLLAAAAGNASSLYTFCLpMGGPSGALLEK</td>
<td>S1193 or T1190</td>
</tr>
<tr>
<td><strong>VCR</strong></td>
<td>678</td>
<td>687</td>
<td>TSGpADVSVDR</td>
<td>S680</td>
</tr>
<tr>
<td></td>
<td>708</td>
<td>730</td>
<td>SKDTNVTpDPDV6GtRSpAFFK</td>
<td>S724</td>
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<tr>
<td></td>
<td>708</td>
<td>730</td>
<td>SKDTNVTpDPDV6GtRSpAFFK</td>
<td>S726</td>
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<tr>
<td></td>
<td>807</td>
<td>823</td>
<td>ENFCqQASSNLpSTEMAR</td>
<td>S818</td>
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<tr>
<td></td>
<td>862</td>
<td>877</td>
<td>LPSGSpGSGLVEASNK</td>
<td>S867</td>
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<tr>
<td></td>
<td>1174</td>
<td>1199</td>
<td>LALTAAGPSFLVpEQLpMPGlGALLEK</td>
<td>S1189</td>
</tr>
<tr>
<td></td>
<td>158</td>
<td>173</td>
<td>pSpTVGTpAVAPIpLSR</td>
<td>S158 or T158</td>
</tr>
<tr>
<td></td>
<td>350</td>
<td>361</td>
<td>TVKEMPpSGEvR</td>
<td>S357</td>
</tr>
</tbody>
</table>

**VCS is a direct target of SnRK2.10, SnRK2.5 and SnRK2.6**

In order to verify which of the proteins that were pulled down with SnRK2.4 and/or SnRK2.10, were direct targets of these kinases in vitro kinase activity assays were performed. We selected VCS, VCR and DCP2 for verification, since they were previously found to be phosphorylated upon osmotic stress (Stecker et al., 2014). Purified recombinant protein kinases SnRK2.4 and SnRK2.10 were used for the in vitro phosphorylation reactions with synthetic peptides from VCS, VSR and DCP2 proteins. Synthetic peptides were designed to contain the phosphopeptides identified in the TAP experiment (Table 2) and by Stecker et al., 2014. Each protein kinase was incubated with 3 peptides representing VCS sequence, one from VCR and DCP2 and, in a separate reaction, with MBP (myelin basic protein) as a positive control. Autophosphorylation of SnRK2.10 and phosphorylation of all three peptides from VCS and a peptide from VCR, but not from DCP2 were detected (Table 3, Fig. S1, Table S2). SnRK2.4 did not phosphorylate itself nor any of
the tested peptides, however it was able to phosphorylate MBP (Table 3, Table S3).

To investigate the redundancy within the SnRK2 family, SnRK2.5 from the same subclass as SnRK2.4 and SnRK2.10 as well as SnRK2.6 from subclass 3 were tested. SnRK2.6 phosphorylated all the peptides, while SnRK2.5 phosphorylated VCR and two out of three VCS peptides, suggesting functional redundancy between SnRK2 protein kinases from subclasses 1 and 3 (Table 3, Table S4-5). Since the peptide corresponding to the DCP2 protein was hardly detectable, probably due to its low solubility, full-length DCP2 recombinant protein was purified and tested in an in vitro kinase assay. Also full-length DCP2 was not phosphorylated in the presence of SnRK2.10, confirming it is not a direct substrate of this kinase in vitro (Table S6). We conclude that VCS and VCR are direct targets of SnRK2.5, SnRK2.6 and SnRK2.10.

Table 3. SnRK2.5, SnRK2.6 and SnRK2.10 phosphorylate VCS peptides. Summary of the in vitro kinase activity assays performed with recombinant protein kinases and synthetic peptides. Position of start and end of the peptides used is relative to the first amino acid in the protein sequence. MBP (myelin basic protein) is a known substrate for SnRK2 protein kinases and was used as a positive control.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Protein kinase</th>
<th>VCS</th>
<th>VCR</th>
<th>DCP2</th>
<th>MBP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SnRK2.4</td>
<td>SnRK2.5</td>
<td>SnRK2.6</td>
<td>SnRK2.10</td>
<td>SnRK2.6</td>
</tr>
<tr>
<td>VCS</td>
<td>636-668</td>
<td>T159</td>
<td>T159</td>
<td>T159</td>
<td>S1155</td>
</tr>
<tr>
<td>VCR</td>
<td>686-701</td>
<td>T269</td>
<td>S29</td>
<td>S29</td>
<td>S29</td>
</tr>
<tr>
<td>DCP2</td>
<td>265-291</td>
<td>-</td>
<td>S119</td>
<td>S119</td>
<td>S119</td>
</tr>
<tr>
<td>MBP</td>
<td>1-354</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Impact of SnRK2 subclass 1 protein kinases on salt-induced transcriptional changes in Arabidopsis seedlings

To further elucidate the functional link between SnRK2 subclass 1 protein kinases, mRNA decay pathways and salt stress, transcriptome profiling of Col-0, snrk2.4, double snrk2.4/2.10 (McLoughlin et al., 2012) and quintuple snrk2.1/2.4/2.5/2.9/2.10 (Fujii et al., 2011) knock-out mutants was performed. To select the most suitable duration of salt stress, a time course experiment with Col-0 seedlings was performed. Ten days-old seedlings grown in liquid media were treated with buffer (mock) or 150 mM NaCl. Kinase activity in the crude extract of proteins from whole seedlings was assessed by in-gel kinase assay with MBP (myelin bovine protein) used as a substrate. Salt treatment resulted in rapid induction of SnRK2.4 and SnRK2.10 activity (37 kDa band) within 30 seconds, which was reduced after 5 minutes and increased again after 24 hours (Fig. 1). Activity of the ABA-dependent SnRK2.2, SnRK2.3, SnRK2.6 (38 kDa) was induced after 10 minutes of salt stress, indicating different activation dynamics of these two SnRK2 subfamilies (Fig. 1). It has to be noted that transcriptional changes that we are interested in can be
a consequence of the action of the potential substrates of SnRK2 and may not be observed immediately after SnRK2 activation. One-hour salt treatment was chosen for transcriptional profiling of Col-0, snrk2.4, double snrk2.4/2.10 and quintuple snrk2.1/2.4/2.5/2.9/2.10 mutants.

Fig. 1. Rapid activation of SnRK2.4 and SnRK2.10 in Arabidopsis seedlings. Ten days-old seedlings grown hydroponically were subjected to a time-course treatment with 150 mM NaCl (left) or 0.5xMS (control, right). In gel kinase assay of crude protein extracts with MBP used as kinase substrate was performed. Band corresponding to the activity of SnRK2.4 and SnRK2.10 present in Col-0 and absent in snrk2.4/2.10 double knock-out mutant, is indicated with an arrow. Coomassie Brilliant Blue staining used as a loading control is presented in the bottom panel.

The upregulation of several genes encoding DREB (DEHYDRATION-RESPONSIVE ELEMENT BINDING PROTEIN), WRKY (WRKY DNA-BINDING PROTEIN) and MYB (MYB DOMAIN PROTEIN) transcription factors in Col-0 upon treatment with 150 mM NaCl confirmed that the seedlings were subjected to salt stress (Table S7; Kreps et al., 2002). Since multiple members of the SnRK2 family in rice, wheat and maize have been shown to be transcriptionally upregulated by salt and osmotic stress (Huai et al., 2008; Kobayashi et al., 2004; Mao et al., 2010; Zhang et al., 2011; Zhang et al., 2010), we checked whether transcription of Arabidopsis SnRK2 genes is regulated by salt stress. The one-hour treatment with 150 mM NaCl resulted in a small upregulation of only SnRK2.5, suggesting that under these conditions most of the Arabidopsis SnRK2 protein kinases are only regulated at the post-transcriptional level, yet we cannot exclude the possibility that they may be transcriptionally regulated at other time points (Fig. S2).

In order to find significant changes in transcript abundance upon salt stress, differential expression analysis was performed using Cuffdiff software. One-hour treatment with 150 mM NaCl resulted in a change in expression of more than two times in 1292 genes, among which 913 were up- and 379 were downregulated (Table S7). Next, we asked what would be the consequences of the mutations in SnRK2 subclass 1 protein kinases for the salt responses and whether any particular group of transcripts would be affected. Comparison of the molecular functions and biological processes controlled by genes up- or
downregulated by salt stress minimum 2 times between 4 genotypes, showed the same distribution for Col-0 and snrk mutants (Fig. S3-S4). This suggests that mutating SnRK2 subclass 1 protein kinases does not influence a specific GO category, but instead affects genes from multiple functional categories. It is also possible that there are categories of genes that would be controlled by all SnRK2 protein kinases, which cannot be captured with the selection of mutants presented in here.

To elucidate what are the salt stress-related transcriptional changes downstream of SnRK2 subclass 1 protein kinases from the list of 1292 genes which expression was affected by salt stress, the genes that were not significantly changed by salinity in each mutant were selected. To assess only the salt stress specific effect, genes for which expression was already altered in the mutant line in control conditions (15, 24 and 65 for snrk2.4, snrk2.4/2.10 and snrk2.1/2.4/2.5/2.9/2.10, respectively) were excluded. The responses of many genes regulated by salt stress in Col-0 were affected by mutations in the subclass 1 SnRK2 genes. Among the genes upregulated by salt stress in Col-0, 109 were not responding in snrk2.4, while the downregulated genes remained unchanged (Table S8). Knocking out both SnRK2.4 and SnRK2.10 resulted in 91 and 77 genes that were up- and downregulated by salt stress, respectively, in Col-0, while they were not responding in the double mutant (Table S9). In the quintuple snrk2.1/2.4/2.5/2.9/2.10 mutant 129 salt stress upregulated genes and 35 downregulated genes in Col-0, were not responding to salt treatment (Table S10). These results imply that 8%, in case of snrk2.4, and 13%, in case of both snrk2.4/2.10 and snrk2.1/2.4/2.5/2.9/2.10, of the genes responding to salt stress in Col-0 do not respond in these mutants.

Comparison of the molecular function of selected SnRK2-dependent genes revealed an increase in genes associated with transporter activity in quintuple mutants compared to single and double mutants, while those annotated with protein binding were over-represented in double and quintuple compared to the single mutant (Fig. 2A). Genes encoding protein kinases represented around 11% of the genes not responding to salt stress in the snrk2.4 mutant, while in snrk2.4/2.10 and snrk2.1/2.4/2.5/2.9/2.10 this was only 6-7% (Fig. 2A). Selected SnRK2-dependent genes associated with the GO categories DNA-dependent transcription and transport were more abundant in double and quintuple mutant compared to the snrk2.4 line (Fig. 2B). Genes involved in responses to stress and responses to biotic and abiotic stimuli were more abundant in the snrk2.4 single mutant than in snrk2.4/2.10 and snrk2.1/2.4/2.5/2.9/2.10, while these annotated with electron transport and energy pathways were present only in double and quintuple mutants (Fig. 2B). Surprisingly, only a small overlap in SnRK2-dependent genes was found between single, double and quintuple mutants (Table S11). Twenty-three of the in total 442 genes influenced by the mutations were shared between the single, double and quintuple mutants (Fig. S5A). The annotation of the overlapping candidates showed different biological functions. Our results
suggest that transcriptional changes that are a consequence of a single SnRK2 protein kinase activity may cover different genes but act on similar processes. Additionally, genes for which expression was affected by salt stress in each mutant, but to a different degree or in an opposite manner than in Col-0 were selected (Table S12-14).

Fig. 2. Molecular function and biological processes controlled by genes selected as putative targets of SnRK2 subclass 1 protein kinases. Candidate genes were selected from genes up- or down-regulated by salt stress minimum 2 times in Col-0 but not affected in snrk2.4, snrk2.4/10 or snrk2.1/4/5/9/10. Functional characterization by annotation for GO Molecular Function (A) and Biological Process (B) was performed with GO annotation tool available at https://www.arabidopsis.org/tools/bulk/go/. Numbers in brackets next to the category description indicates percentage of the number of annotations in this category from the numbers of total annotations within the candidates list for each mutant. Asterisks indicate categories that showed different representation between mutants.
Expression of several osmotic stress-induced genes previously described to be dependent on SnRK2.2, SnRK2.3 and SnRK2.6 (RD29A, RD29B, RD26, NCED3, PKS5, KIN2, AREB1, HAI1, COR15A, DREB2A, ABI1; Fujii et al., 2011; Fujita et al., 2009; Yoshida et al., 2002) was not affected by any of the mutations tested here, indicating a separation of the effect of subclass 1 and 3 SnRK2 kinases in the regulation of the expression of, at least, these genes (Table S8-S10).

Due to some variation between biological replicates, that we observed for several genes from the list of the putative SnRK-dependent genes (Table S8-S10, Table S12-S14), only those of which coefficient of variation between biological replicates was lower or equal to 0.4 were selected for further analysis. This selection resulted in 62, 127 and 169 candidate genes of which response to salt stress was different from Col-0 in snrk2.4, snrk2.4/2.10 and snrk2.1/2.4/2.5/2.9/2.10, respectively (Table S15-S17). Based on the highest difference in expression response to salt between Col-0 and mutants (calculated as the ratio between the response to salt in the mutant and the response to salt in Col-0), 10 candidate genes were selected for further validation.

SnRK2 subclass 1 protein kinases regulate the expression of aquaporins, a bZIP transcription factor, beta glucosidase and a cytochrome P450

Expression of 10 selected candidates was verified by qPCR using RNA extracted from an independent biological experiment. To investigate whether salt-induced changes in transcript abundance downstream of SnRK2 subclass 1 kinases are also dependent on mRNA decapping and 5’ mRNA decay, a vcs1 mutant (point mutation in Ler background) and an xrn4-5 Col-0 knock-out mutant were analyzed as well. XRN4 is the only 5’->3’ exoribonuclease identified so far in Arabidopsis. Changes upon salt stress in the expression of two genes (MP3K17 and MP3K18,) were confirmed by qPCR for Col-0, but not for the snrk mutants, while the expression of two other genes (EXO, ATSZF1) was consistent with RNAseq data in both Col-0 and snrk mutants but the decreased response in the mutants was not significant (Fig. S6).

The salt-control ratio of the expression of five other selected genes was confirmed by qPCR to be lower in all snrk mutants than in Col-0 (Fig. 3A-E). PLASMA MEMBRANE INTRINSIC PROTEIN 2;5 (PIP2;5) showed 3 times upregulation by salt in Col-0, while in single, double and quintuple snrk mutants this ratio was around 1 (Fig. 3A). No differences in PIP2;5 expression between genotypes were observed for control conditions, while a severe decrease in comparison to Col-0 was seen for salt stress in the snrk mutants. Similar salt-control ratios of PIP2;5 expression were observed for snrk2.4, snrk2.4/2.10 and quintuple snrk2.1/2.4/2.5/2.9/2.10 mutants, suggesting that induction of PIP2;5 expression upon salinity may depend on SnRK2.4 (Fig. 3A). Another gene from the same family, PIP2;3, was also upregulated by salt in Col-0 (Fig. 2B). No differences in PIP2;3 expression between
genotypes were observed for control conditions, while upon salt treatment the differences in snrk mutants were subtle (Fig. 3B). Since the response to salt of snrk2.4/10 were significantly different from Col-0 we conclude that the expression of PIP2;3 rely at least on redundant action of SnRK2.4 and SnRK2.10 (Fig. 3B). Expression of BETA GLUCOSIDASE 6 (BGLU6) on salt stress, but not under control conditions, was reduced in snrk2.4/2.10 and snrk2.1/2.4/2.5/2.9/2.10, suggesting that BGLU6 upregulation by salt observed in Col-0 requires SnRK2.4 and SnRK2.10 signaling (Fig. 3C). Induction by salt of the expression of CYTOCHROME P450, FAMILY 79, SUBFAMILY B, POLYPEPTIDE 2 (CYP79B2) was observed in Col-0, but not in snrk mutants and gradual decrease of the response to salt was seen between single, double and quintuple mutant (Fig. 3D). The differences of CYP79B transcript abundance in the presence of salt were not significant, but response to salt was 3 times lower in snrk2.4/2.10 and snrk2.1/2.4/2.5/2.9/2.10 mutants than in Col-0, which suggests that CYP79B2 induction by salt may be dependent on SnRK2 subclass 1 protein kinases (Fig. 3D). Abolished induction of CYSTEINE-RICH RECEPTOR-LIKE PROTEIN KINASE 45 (CRK45) by salt was observed for all snrk mutants, but the difference from Col-0 was only significant for snrk2.4/2.10 (Fig. 2E). Because of the high variation of biological replicates, results of the snrk2.1/2.4/2.5/2.9/2.10 line are not conclusive for this gene. Nevertheless CRK45 expression seems to be dependent on SnRK2.4 and SnRK2.10 (Fig. 3E). Results of RNAseq suggested that BASIC LEUCINE-ZIPPER 3 (BZIP3) gene is downregulated by salt (Table S7). The qPCR data did not support that, since the expression of BZIP3 was not regulated by salt (Fig. 3F). However in all snrk mutants
BZIP3 expression was lower than in Col-0 on salt stress, but not in the control conditions, suggesting that SnRK2 subclass 1 kinases may indirectly control salinity-dependent BZIP3 expression (Fig. 3F).

To check whether observed changes in the abundance of the aforementioned transcripts could be a consequence of the VCS phosphorylation by SnRK2 subclass 1 protein kinases and possibly by alterations in 5’ mRNA decay, changes in the expression of the same transcripts were checked in vcs1 and xrn4-5 mutant. Differences in the xrn4-5 mutant were similar to these observed in snrk mutants for PIP2;5, CYP79B2, BGLU6 and BZIP3, but not for CRK45, suggesting that some changes in transcript abundance observed in snrk mutants may be also linked to 5’mRNA decay (Fig. 3). Since in Ler the expression of most of the candidates did not respond to salt stress in the similar way as in Col-0, no conclusions could be drawn for these transcripts (Fig. 3A, B, D, F). For PIP2:3 and CRK45, salt-induced expression was found also in the Ler background, and was not affected in the vcs1 mutant, implying that SnRK2 subclass 1 protein kinases regulate their expression via a pathway that does not include VCS (Fig. 3C, E).

Components of 5’ mRNA decay pathway contribute to root responses to salt stress

SnRK2.4 and SnRK2.10 were shown previously to have a positive role in elongation of the main root and in lateral root emergence under salt stress, respectively (McLoughlin et al., 2012). Our kinase activity assays suggest that VCS, as a direct substrate of SnRK2.10, may act downstream in the same pathway. Salt-induced changes in root growth of vcs1 and xrn4-5 mutants were tested by transferring 4 days-old seedlings germinated on half-strength MS medium to media supplemented with 75 and 125 mM NaCl. Main root length of vcs1 was higher than wild type (Ler) on all conditions tested, but increase in lateral root density and total root size was observed on salt stress only (Fig. 4A). The xrn4-5 knock-out mutant showed lower main root length, lateral root density and total root size than Col-0 on 125 mM NaCl, with no differences under control conditions (Fig. 4B). Thus, the xrn4-5 mutant phenocopies the snrk2.4 and snrk2.10 mutants, while a mutation in vcs1 results in an opposite phenotype. Together these results suggest that VCS and XRN4 contribute to salt-induced modulation of root growth, but in a different manner and further characterization is necessary to link them to SnRK2.4 and SnRK2.10 signaling pathways.
Fig. 4. Components of 5’ mRNA decay are involved in root responses to salinity. Four days-old seedlings of (A) Ler and vcs1 and Col-0 and (B) xrn4-5 were transferred to media supplemented with 0, 75 or 125 mM NaCl. Main root length (MRL) lateral root density (LRD) and total root size (TRS) were quantified from 8 and 10 days-old seedlings for control and stress, respectively. Values presented are averages for 18 replicates. Error bars represent standard error. Statistical comparison was done by 2 way-Anova followed by LSD post-hoc test (p<0.05). Different letters indicate significant differences.

Discussion

SnRK2.4 and SnRK2.10 protein kinases are involved in early responses to osmotic stress and salinity. Their rapid activation within the first minutes after salt treatment is independent of ABA, and SnRK2.4 and SnRK2.10 have been shown to promote root growth in the presence of salt (McLoughlin et al., 2012). To understand the ABA-independent mechanism of early salt stress signaling leading to regulation of root growth we aimed to identify components of SnRK2.4 and SnRK2.10 protein kinases pathway.

In planta co-purification experiments showed that both kinases can physically interact with proteins involved in 5’ mRNA decay (Table 1, Table S1). Degradation of mRNA from 5’ end requires removal of the 5’cap by the decapping complex followed by the digestion by 5’-3’ exoribonuclease XRN4. Two of the proteins interacting with SnRK2.4 and SnRK2.10, VCS (VARICOSE) and DCP2 (DECAPPING 2), are members of the mRNA decapping complex (Xu et al., 2006). DCP2 has decapping activity, while VCS acts as a scaffold protein between DCP2 and third member of this complex, DCP1 (DECAPPING1), which functions as a DCP2 activator (Goeres et al., 2007; Xu et al., 2006). Homozygous knock-out mutants of decapping complex members are lethal, while heterozygous lines or point mutants show severe developmental alterations, implying the importance of 5’mRNA decapping (Deyholos et al., 2003; Goeres et al., 2007; Xu et al., 2006). Removal of 5’ cap structure leaves the mRNA unprotected from the exoribonucleitic activity of
XRN4 (Kastenmayer and Green, 2000). Other proteins identified as putative SnRK2.4 and SnRK2.10 interactors, VCR (VARICOSE-RELATED), ATRRP44B (RRP44 HOMOLOG B) and TOPOISOMERASE II-ASSOCIATED PROTEIN PAT1H1 (Table 1) are also involved in mRNA metabolism processes, but their function is not well understood yet (Deyholos et al., 2003; Kumakura et al., 2013; Roux et al., 2015; Xu et al., 2006; Zhang et al., 2010).

The molecular function of identified proteins suggests they act downstream of SnRK2.4 and SnRK2.10 and are putative substrates of these protein kinases. All of the identified aforementioned putative SnRK2.4 and SnRK2.10 interactors have been shown to colocalize to P bodies, cytoplasmic foci, which are the site of mRNA decay (Maldonado-Bonilla, 2014; Sheth and Parker, 2003). This may suggests that not all of the identified proteins interact directly with SnRK2.4 and SnRK2.10. Moreover in our co-purification assay in vivo phosphorylation sites were found only for VCS and VCR (Table 2). The phosphorylation of VCS peptides: TPpSADYSVDR, ESITSApSpSVAQALSR, and TSGLPSQTSGAGSAYATLPQLPSPR, and the TSpSADYFYVR peptide from VCR have previously been shown to be affected by osmotic stress (Stecker et al., 2014). Direct phosphorylation of VCS and VCR by SnRK2.10 was confirmed in in vitro kinase activity assay (Table 3). Even though SnRK2.4 was able to phosphorylate MBP, the peptide corresponding to its known autoactivation site was not detected (Stecker et al., 2014; Vlad et al., 2010; Table 3, Table S3). Several MBP phosphorylation sites were found in the reaction with SnRK2.10 and only one for SnRK2.4, suggesting that these kinases have different activity. A similar difference in activity was already observed in Arabidopsis protoplasts treated with mannitol, but not with NaCl (Boudsocq et al., 2004). No phosphorylation of VCS and VCR peptides has been detected in the presence of SnRK2.4 in the in vitro assay, but results obtained in planta suggest that SnRK2.4 and SnRK2.10 can redundantly phosphorylate VCS and VCR in response to salt stress (Table 2, Table 3). It is also possible that SnRK2.4 phosphorylates VCS at different serine residues than SnRK2.10, which were not covered by the peptides used for in vitro assay, but were identified with in planta experiment (Table 2). Another kinase from the SnRK2 subclass 1 subfamily, SnRK2.5, was also able to phosphorylate 2 out of 3 tested VCS peptides, but not VCR, which could possibly be because its lower activity compared to SnRK2.10 (Table 3, Table S4). A previous phosphoproteomic study identified VCS as a putative substrate for ABA-dependent SnRK2 kinases, yet this interaction was not confirmed (Umezawa et al., 2013). In our in vitro assay SnRK2.6 was able to phosphorylate VCS and VCR at the same residues as SnRK2.10 (Table 3). Together our data suggest that VCS and VCR are substrates for both ABA-dependent and independent SnRK2 protein kinases. VCR is a non-functional homolog of VCS (Xu et al., 2006) and similar phosphopeptides were found for both proteins, implying that VCS and VCR
are likely to be targets of SnRK2.4, SnRK2.5, SnRK2.6 and SnRK2.10, but only phosphorylation of VCS may have biological relevance. We hypothesize that other co-purified proteins are not substrate for SnRK2.4 and SnRK2.10, but rather indirect interactors, however this still requires confirmation.

Another protein that co-purified with SnRK2.4, but not SnRK2.10, is the transcription factor ABA-RESPONSIVE ELEMENT BINDING PROTEIN 3 (AREB3), which was previously shown to be a substrate of SnRK2.6. This could again suggest functional overlap between members of SnRK2 subclass 1 and 2 families, but might also present an interaction of AREB3 via SnRK2.6, which was also identified in the TAP experiment (Table 1). Moreover, co-purification of other SnRK2 protein family members (SnRK2.6, SnRK2.5, SnRK2.9) may suggest that these kinases can regulate each other’s activity (Table 1).

Altered sensitivity to osmotic and salt stress of mutants in mRNA metabolism factors suggests that mRNA decapping and its subsequent 5’ decay regulate plant responses to these stresses (Kawa and Testerink, 2016). It remains unknown whether phosphorylation of VCS can modulate assembly or activity of the decapping complex. Two serine residues in VCS, S660 and S692, were also found to be phosphorylated in control conditions (de la Fuente van Bentem et al., 2008). The phosphorylation of VCS in response to mannitol occurred at multiple serine residues, with some sites more and some less abundant upon osmotic stress (Stecker et al., 2014). Most of the VCS phosphosites identified here (Table 2) or in previous studies were found in the region in between two domains responsible for protein-protein interactions (Stecker et al., 2014; Umezawa et al., 2013; Wang et al., 2013). Importantly, phosphorylation of other component of Arabidopsis decapping complex DCP1 and complex of Sm-like proteins LSM1-7, acting as a decapping activator, as well as of human DCP1a and yeast DCP2 was linked to the responses to stress responses (Xu and Chua, 2012). Phosphorylation of VCS upon osmotic stress has been suggested to depend on SnRK2, MAP3K or MAP4K kinases and here we confirm that VCS is phosphorylated by at least SnRK2.5, SnRK2.6 and SnRK2.10 (Table 3).

To explore further the molecular events downstream of SnRK2 subclass 1 protein kinase signaling we analyzed the salt-induced transcriptional changes in snrk2.4, snrk2.4/2.10 and snrk2.1/2.4/2.5/2.9/2.10. Salt treatment resulted in differential expressions of genes of many functions and involved in several biological processes (Table S7), but the role of single SnRK2 genes seems not to be restricted to specific molecular processes (Fig. S3-S4).

From 10 selected SnRK2-dependent candidate genes, expression of 5 genes, upregulated by salt stress in Col-0, were not induced, or to a lower extent, in the mutants (Fig. 3). Expression of water channel PIP2;5 seems to be dependent solely on SnRK2.4, while for PIP2;3 both SnRK2.4 and SnRK2.10 may be required (Fig. 3A-B). PIP2;3 have been shown to be involved in LR emergence, suggesting its involvement in the same process as SnRK2.10 (Peret 57
et al., 2012), PIP proteins contribute to root cell hydraulic conductivity making them possible downstream targets of SnRK2s signaling under osmotic and salt stress (Alexandersson et al., 2010).

SnRK2.4 and SnRK2.10 were also required for salinity-dependent induction of flavonol glucosyltransferase BGLU6 (Fig. 3C), which by regulation of flavonol metabolism can contribute to scavenging reactive oxygen species accumulating upon salt stress (Agati et al., 2012; Ishihara et al., 2016). Also the salt-induced upregulation of cytosolic receptor-like kinase (RLK) CRK45 was abolished in snrk2.4/2.10 and snrk2.1/2.4/2.5/2.9/2.10 mutants (Fig. 3E). Although CRK45 was previously implicated in ABA signaling (Tanaka et al., 2012), our results indicate that it may also be involved in ABA-independent responses to salt stress. Another gene for which induction by salt stress was dependent on SnRK2.4 and SnRK2.10, encodes for the cytochrome P450 protein CYP79B2 (Fig. 3D). Remarkably, CYP79B2 was expressed at the sites of lateral root formation and similar localization was found for SnRK2.10 (Ljung et al., 2005; McLoughlin et al., 2012). CYP79B2, via the conversion of tryptophan to indole-3-acetaldoxime, is involved in local biosynthesis of auxin, the key hormone regulating root development (Ljung et al., 2005; Mikkelsen et al., 2000). Expression of BZIP3, not changed by salinity in Col-0, was downregulated in all snrk mutants tested, suggesting that in the presence of salt SnRK2 subclass 1 can activate an unknown negative regulator of BZIP3 expression (Fig. 3F).

Data obtained so far with a vcs1 mutant imply that SnRK2-dependent upregulation of PIP2;3 and CRK45 is guided via phosphorylation of proteins other than VCS (Fig. 3B, E). Induction of PIP2;5, CYP79B2, BGLU6 and BZIP3 was abolished not only in snrk mutants, but also in xrn4-5 line, which may suggest that they are part of SnRK2.4 and SnRK2.10 signaling pathway involving regulation of the 5’ mRNA decay. On the other hand XRN4 has been shown to affect transcripts abundance not only via their degradation but also indirectly and observed differences in selected mRNAs may not be the effect of 5’->3’ cleavage (Rymarquis et al., 2011). Moreover it is still unknown whether other regulators acting downstream of SnRK2s but upstream of selected mRNAs are also involved in observed changes. It also has to be noted that 5’ mRNA decay processes are very dynamic and further characterization of the abundance of transcripts selected here, as well as their stability, is required.

Results presented here suggest that SnRK2 protein kinases activated via different mechanisms can show functional redundancy. However phosphorylation of VCS by SnRK2.6 still needs confirmation in planta. P bodies, sites of the VCS localization resemble punctate structures, to which SnRK2.4 relocates in root cells upon salt treatment (Goeres et al., 2007; McLoughlin et al., 2012), but no subcellular localization was reported for SnRK2.6 or SnRK2.5 so far. Expression of two aquaporins, PIP2;5 and PIP2;3 were shown to be dependent on SnRK2.4 and/or SnRK2.10 (Fig. 3). Interestingly yet another aquaporin PIP2;1 was phosphorylated by SnRK2.6
suggesting that function of aquaporins can be regulated by SnRK2 kinases via two mechanisms; ABA-dependent via direct phosphorylation and indirectly by non-ABA dependent SnRK2s. Expression of a subset of selected salt-induced genes was altered to the same extent in snrk2.4/2.10 and snrk2.1/2.4/2.5/2.9/2.10, as well as in an xrn4 mutant, suggesting regulation of the expression of these genes via 5’ mRNA decay could rely mainly on SnRK2.4 and SnRK2.10 action (Fig. 3). Similarly, activity of SnRK2.4 and SnRK2.10 was higher than other members of subclass 1 (Fig. 1), suggesting higher relevance of SnRK2.4 and SnRK2.10 signaling over the other class 1 members. On the other hand several candidate transcripts were found for snrk2.1/2.4/2.5/2.9/2.10, but not single and double mutants, implying that SnRK2.1, SnRK2.5 and SnRK2.9 signaling can also govern salt-induced changes in gene expression (Fig. S5B, Table S11).

Our results suggest that SnRK2.4 and SnRK2.10 can regulate root growth in the presence of salinity by targeting 5’ mRNA decay machinery and regulation of the expression of genes involved in water transport and auxin biosynthesis, yet the exact connection between these processes remains to be discovered.

Material and Methods

Identification of SnRK2.4 and SnRK2.10 interactors

The coding regions of SnRK2.4 and SnRK2.10 were cloned under CaMV 35S promoter for fusion with GSrhino tag in pH7m24GW2 vector for N- and pH7m34GW2 for C-terminal fusion with Multisite Gateway cloning as described in (Van Leene et al., 2015). Arabidopsis PSB-D cell suspension cultures were transformed and tandem affinity purification (TAP) of SnRK2.4 and SnRK2.10 protein complexes was performed according to the protocol described in (Van Leene et al., 2015). Eluted proteins were identified on LTA OrbitrapVelos with two technical replicates per bait. Proteins identified with at least two peptides were considered as significant. Most abundant background proteins were subtracted and final list of the putative interactors is presented in Table 1.

Protein expression and purification

GST fusions were obtained by cloning full-length coding sequence of SnRK2.4, SnRK2.5, SnRK2.6 and SnRK2.10 into pGEX4T1 and DCP2 into pGEX-KG Gateway vector. All constructs were transformed to E.coli BL21 DE3 and their expression was induced for 3 hours with 1mM IPTG in 18°C. Recombinant proteins were purified with GST-Sepharose beads (GE Healthcare, UK) as described in (Julkowska et al., 2015).

In vitro kinase activity assays

Peptides harboring putative phosphorylation sites were synthesized by GenScript (China). Sequences of used peptides can be found in Table 3. 1 µM of each peptide was incubated with 0.1 µM of recombinant protein kinase in kinase reaction buffer (50 mM Tris-HCl, pH 7.5, 2 mM MgCl₂, 1 mM DTT, 1 mM ATP) in a final volume 60 µl for 6 hours in 30°C. 20 µl of each reaction was used for direct trapping and collection of the synthetic peptides on 8 µg capacity OMIX RP tip (Agilent Technologies). The trapped peptides were eluted in 10 µl 50% acetonitrile (ACN), 0.1% trifluoroacetic acid (TFA) and 3-5 µl fraction was dried in a speedvac and reconstitute in 6 µl 2% acetonitrile, 0.1% TFA for the analysis with LC-MS. Remaining 40 µl of each reaction was used for in-solution digestion. Samples were reduced with 10 mM DTT for 30 minutes at 60°C followed by alkylation with 20 mM iodoacetamide for 30 minutes at room
temperature in darkness. An overnight digestion with 2 µg trypsin (Sigma) was performed at 37 °C, stopped with TFA and peptides were collected with 50% ACN, 0.1% TFA on 8 µg capacity OMIIX RP tip (Agilent Technologies), dried and reconstituted in 6 µl 2% ACN, 0.1% TFA for the analysis with LC-MS. For the kinase activity assays with full-length protein as a substrate, 0.4 µg of recombinant protein kinase and 2 µg of the substrate were used for the same reactions as with peptides in a total volume of 30 µl, digested with trypsin, collected and analyzed as described above. Myelin basic protein (MBP, Upstate) was used as a substrate for a positive control of protein kinase activity.

Mass spectrometry analyzes
Mass spectrometry analyzes were done with the amaZon Speed Iontrap with a CaptiveSpray ion source (Bruker) coupled to an EASY-nLC II (Proxeon, Thermo Scientific) chromatographic system. Peptide samples were injected and separated with an eluent flow of 300 nL x min⁻¹ on an Acclaim PepMap100 (C18 75µM 25cm Dionex, Thermo Scientific) analytical column combined with an Acclaim PepMap100 pre-column (C18 100µM 2cm Dionex, Thermo Scientific) using a 30 minute gradient of 0–50% ACN and 0.1% formic acid. Peptide precursor ions above a predefined threshold ion count were selected for low-energy collision-induced dissociation (CID) to obtain fragmentation spectra of the peptides. Technical replicates were performed with electron-transfer dissociation (ETD). MS/MS data were processed with Data Analysis software (Bruker), and used for database searching with Mascot software (Version 2.5.1) in a custom-made database containing all SnRK protein kinases, MBP and the synthetic peptides sequence information. Searches were simultaneously performed against a common contaminants database (compiled by Max Planck Institute of Biochemistry, Martinsried) to minimize false identifications. Mascot search parameters were as follows: a fixed modification of carbamidomethyl for cysteine, variable modification of oxidized methionine and Phospho(ST), trypsin with the allowance of one missed cleavage, peptide charge state +2, +3, and +4. Peptide and MS/MS mass error tolerances were 0.3 Da for ESI-trap or ETD-trap. For the sample with synthetic peptides no fixed modification was applied. The identified phosphopeptides were verified by manual inspection of MS/MS spectra in the raw data using the Data Analysis software.

In-gel kinase activity assay
Arabidopsis thaliana seeds were surface sterilized with 20 ml thin bleach and 600 µL 37.5% HCl for 3 hours and then placed for 1.5 hour in laminar flow to evaporate chlorogenic acid. Seeds were stratified for 72 hours at 4°C and grown under long day conditions (21°C, 70% humidity, 16/8 light/dark cycle) in 100 ml liquid media containing 0.5xMurashi Skoog basal salt, 0.5% sucrose, 1% M.E.S. monohydrate, pH 5.8 (KOH) with shaking (120 rpm). Ten days-old seedlings were grown at 21°C, 70% humidity, with washing buffer (25 mM Tris-HCl pH 7.5, 5 mM EDTA, 5 mM EGTA, 25 mM NaF, 1 mM NaVO₄, 50 mM β-glycerophosphate, 1x complete protease inhibitor cocktail (Promega) and spun down at 20 000 g for 30 minutes. Protein concentration was determined with Bradford protein assay (Bio-Rad). 50 µg of crude protein extract was separated on 12% polyacrylamide gel containing 0.2mg/ml of myelin basic protein (MBP, Upstate). Gels were washed 3 times for 30 minutes at room temperature with washing buffer (25 mM Tris-HCl pH 7.5, 0.5 mM DTT, 0.1 mM Na₂VO₄, 5 mM NaF, 0.5mg/ml BSA, 0.1% Triton X-100) and then twice for 30 minutes and overnight at 4°C in renaturation buffer (25 mM Tris-HCl pH 7.5, 0.5 mM DTT, 0.1 mM Na₂VO₄, 5 mM NaF). Gels were incubated in reaction buffer (25 mM Tris-HCl pH 7.5, 2 mM EGTA, 12 mM MgCl₂, 1 mM DTT, 1mM, 0.1 mM Na₂VO₄) for 30 min at 37°C and then brought to reaction buffer containing 25 µM ATP and 50 µCi ³²P γ-ATP for 1 hour. Gels were washed 6 times in 1% Na₂HPO₄, 5% TCA, followed by 30 minute incubation in 3% glycerol, dried overnight and exposed to Storage Phospho Screen (Fuji) for two weeks and scan by phosphoimager (Typhoon FLA 7000, GE Healthcare, Uk).
Transcriptome profiling

Arabidopsis thaliana seeds were surface sterilized with 20 ml thin bleach and 600 µL 37.5% HCl for 3 hours and then placed for 1.5 hour in laminar flow to evaporate chlorine gas. Seeds were stratified for 72 hours at 4°C and grown under long day conditions (21°C, 70% humidity, 16/8 light/dark cycle) in 100 ml liquid media containing 0.5xMurashige-Skoog basal salt, 0.5% sucrose, 1% M.E.S. monohydrate, pH 5.8 (KOH) with shaking (120 rpm). Ten days-old seedlings were treated with 150 mM NaCl in 0.1xMS media (salt stress) or 0.1xMS media (control) for time indicated separately per experiment. Seedlings were dried with paper towel and snap frozen in liquid nitrogen. 100 mg of tissue was ground and total RNA was extracted using Plant RNA extraction kit (Qiagen) according to manufacturer’s instructions. RNA quality determination, library preparation and sequencing with Illumina HiSeq 2500 was performed by Eurofins Genomics (Germany). The reads were trimmed and cleaned using FastQC and Trimmomatic and then aligned to Col-0 genome from TAIR 10.30 database using TopHat algorithm (Kim et al., 2013). Transcripts were assembled and their abundance was quantified using Cufflinks where significant changes in transcript abundance between samples were detected with Cuffdiff (Trapnell et al., 2012). For the selected candidates, expression levels were confirmed by qPCR on RNA extracted from an independent biological experiment performed under the same conditions as used for RNAseq analysis. cDNA was synthesized with ReverAid Kit (Fermentas) and 5 µl were used for each reaction with Eva-Green kit (Solis Biodyne). Three biological replicates were used per line and two technical replicates were made. The sequences of primers are indicated in Table S18. The transcript level was normalized by expression of the reference gene MON1 (At2G28390) according to the following formula: ΔCt=2^[(Ct candidate gene)/2(Ct reference gene)].

Root System Architecture Assay

Seeds were surface sterilized with 20 ml thin bleach and 600 µL 37.5% HCl for 3 hours followed by 1.5 hour in laminar flow to evaporate chlorine gas. Seeds were stratified in 0.2% agar at 4°C in the dark for 72 h. Seeds were germinated on half-strength Murashige-Skoog medium supplied with 0.5% sucrose, 0.1% M.E.S. monohydrate and 1% agar, pH 5.8 (KOH). Seeds were germinated on vertically positioned plates (70° angle) under long day conditions (21°C, 70% humidity, 16/8h light/dark cycle). For days-old seedlings were transferred to 0.5xMS media supplemented with 0, 75 or 125 mM NaCl. Root System Architecture of 8 and 10 days-old seedlings from control and salt stress conditions, respectively, was quantified with EZ-Rhizo Software (Armengaud et al., 2009). Three independent biological experiments with 18 replicates were performed.

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Supplemental Materials

Fig. S1. Spectra of the phosphorylated synthetic peptides identified in *in vitro* kinase activity assays. Presented spectra correspond to the peptides phosphorylated by SnRK2.10 as listed in Table 3.

Fig. S2. Transcriptional regulation of SnRK2 protein kinases under salt conditions. Ten days-old seedlings grown in liquid 0.5xMS medium were subjected to control (0.1xMS) and salt treatment (150 mM NaCl in 0.1xMS) for one hour. Values presented are average fragments per kilobase of transcript per million mapped reads (fpkm) from 3 biological replicates. Error bars represent standard error (SEM). Significant changes according to Student-t test are denoted with asterisk (p<0.05).
Fig. S3. Molecular function of genes up- or downregulated by salt stress minimum 2 times in Col-0, snrk2.4, snrk2.4/10 and snrk2.1/4/5/9/10. Functional characterization by annotation for GO Molecular Function was performed with GO annotation tool available at https://www.arabidopsis.org/tools/bulk/go/. Numbers in brackets next to the category description indicates percentage of the numbers of annotations in this category from the numbers of total annotations within the genotype.
Fig. S4. Biological process of genes up- or downregulated by salt stress minimum 2 times in Col-0, snrk2.4, snrk2.4/10 and snrk2.1/4/5/9/10. Functional characterization by annotation for GO Biological Process was performed with GO annotation tool available at https://www.arabidopsis.org/tools/bulk/go/. Numbers in brackets next to the category description indicates percentage of the numbers of annotations in this category from the numbers of total annotations within the genotype.

Fig. S5. Overlap of number of candidate genes with altered salt-induced expression in snrk2.4, snrk2.4/10 and snrk2.1/4/5/9/10 in comparison to Col-0.
Fig. S6. Expression of EXO, ATSF1, MP3K17 and MP3K18 shown as a ratio of normalized expression on salt stress and control condition. Values presented are averages of normalized expression levels of 3 replicates and error bars denote standard error. Statistical comparison was done by one-way ANOVA followed by LSD post-hoc test (p<0.05). Different letters indicate significant differences. Lack of the letters within one graph means lack of the significant differences. Vcs1 mutant should be compared with its background line Ler, while all the other mutants are in Col-0 background.

Supplemental Data Available On-line

Table S1. List of putative SnRK2.4 and SnRK2.10 interactors and their features. Proteins identified by LC-MS/MS after TAP procedure from Arabidopsis suspension cultures. GSgreen N- and C-terminal fusions with SnRK2.4 and SnRK2.10 were used as baits with two technical replicates. Table presents proteins identified with at least 2 peptides for each sample. Green font indicates baits, while blue stands for proteins that are common background, but can still be possible interactors. Normalized Spectral Abundance Factor was based on the total number of spectra identifying each protein, normalized by the length of the protein and the total number of identified spectra in the sample.

Table S2. Peptides identified with MS/MS analysis of the in vitro kinase activity assay with SnRK2.10 and MBP as a substrate. Peptide in italics was also found in MBP protein in the absence of the kinase.

Table S3. Peptides identified with MS/MS analysis of the in vitro kinase activity assay with SnRK2.4 and MBP as a substrate. Peptide in italics was also found in MBP protein in the absence of the kinase.

Table S4. Peptides identified with MS/MS analysis of the in vitro kinase activity assay with SnRK2.5 and MBP as a substrate. Peptide in italics was also found in MBP protein in the absence of the kinase.
Table S5. Peptides identified with MS/MS analysis of the in vitro kinase activity assay with SnRK2.6 and MBP as a substrate. Peptide in italics was also found in MBP protein in the absence of the kinase.

Table S6. Peptides identified with MS/MS analysis of the in vitro kinase activity assay with SnRK2.10 and DCP2 as a substrate.

Table S7. List of genes with expression changed by salt. Ten days old seedlings grown in liquid cultures were treated with mock or 150 mM NaCl for 1 hour. List includes genes with expression changed minimum 1-fold upon salt treatment.

Table S8. List of genes with expression changed in Col-0 upon 1 hour treatment with 150 mM NaCl, but not affected in snrk2.4 mutant. Ten days old seedlings grown in liquid cultures were treated with mock or 150 mM NaCl for 1 hour. List includes genes with expression changed minimum 1-fold comparing to Col-0.

Table S9. List of genes with expression changed in Col-0 upon 1 hour treatment with 150 mM NaCl, but not affected in snrk2.4/10 mutant. Ten days old seedlings grown in liquid cultures were treated with mock or 150 mM NaCl for 1 hour. List includes genes with expression changed minimum 1-fold comparing to Col-0.

Table S10. List of genes with expression changed in Col-0 upon 1 hour treatment with 150 mM NaCl, but not affected in snrk2.1/2.4/5/9/10 mutant. Ten days old seedlings grown in liquid cultures were treated with mock or 150 mM NaCl for 1 hour. List includes genes with expression changed minimum 1-fold comparing to Col-0.

Table S11. Overlap of the genes with expression changed by salt stress in snrk2.4, snrk2.4/10 and snrk2.1/4/5/9/10.

Table S12. List of genes which expression was regulated in snrk2.4 mutant, but to a different degree or in an opposite manner than in Col-0.

Table S13. List of genes which expression was regulated in snrk2.4/10 mutant, but to a different degree or in an opposite manner than in Col-0.

Table S14. List of genes which expression was regulated in snrk2.1/4/5/9/10 mutant, but to a different degree or in an opposite manner than in Col-0.

Table S15. Transcripts selected from the low variance subset of genes as a putative targets of molecular events downstream SnRK2.4.

Table S16. Transcripts selected from the low variance subset of genes as a putative targets of molecular events downstream SnRK2.4 and SnRK2.10.

Table S17. Transcripts selected from the low variance subset of genes as a putative targets of molecular events downstream all SnRK2 subclass 1 protein kinases.

Table S18. List of the primers used for qPCR.